A NEW ANTITUMOR ANTIBIOTIC, KIDAMYCIN. I
ISOLATION, PURIFICATION AND PROPERTIES OF KIDAMYCIN

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A strain of Streptomyces, designated as No. 2-89, newly isolated from a soil sample was very closely related in its cultural and morphological characteristics to variant strains of *Streptomyces phaeoverticillatus* which originally produced iyomycins. This strain was named as *S. phaeoverticillatus* var. *takatsukiensis* after the location, Takatsuki-shi, Osaka, Japan, where the soil sample yielding the strain No. 2-89 was collected. When the strain of *S. phaeoverticillatus* var. *takatsukiensis* was fermented in the culture medium containing an anthraquinone sulfonate compound, a new antimicrobial and antitumor antibiotic named kidamycin, was produced. Kidamycin was obtained as orange red crystals, and differed from iyomycin B_1 by its toxicity, melting point, and infrared absorption spectrum. The taxonomy of the strains, fermentation, isolation, and physico-chemical and biological properties of kidamycin were investigated.

HATA *et al.*[1] discovered a new antibacterial and antitumor antibiotic, iyomycin, produced by *Streptomyces phaeoverticillatus*[2], and reported that iyomycin was a complex consisting of a high molecular substance and a small amount of a low-molecular substance, iyomycin B, and that iyomycin B was further separated into components B_1 to B_5[3]. Among them, iyomycin B_1 and its acetyl derivative exerted a marked inhibitory activity upon experimental animal tumors[4]. Afterwards, the iyomycin-producing strain gradually had diminished the ability to produce iyomycin B. Accordingly, much efforts have been made to recover the ability of the strain itself to produce iyomycin B as well as to increase the production of iyomycin B by the addition of various substances to the fermentation medium.

As a result, it was discovered that when a variant strain No. 1-125 or No. 613-15 of *S. phaeoverticillatus* was cultured in the medium containing an anthraquinone sulfonate compound several antimicrobial and antitumor antibiotics were produced in good yield.

On the basis of morphological findings, these variant strains never produced secondary whirls with spirals (*biverticillus spira*, characteristic to *S. phaeoverticillatus*), but produced mainly primary whirls with spirals (*monoverticillus spira*). On the other hand, the new strains which produced iyomycin B had been sought among

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Table 1. Comparison between *Streptomyces phaeoverticillatus* and *S. phaeoverticillatus* var. *takatsukiensis*

Medium	*S. phaeoverticillatus* var. *takatsukiensis*			*S. phaeoverticillatus*[2]
	No. 613-15	No. 1-125	No. 2-89	
Starch agar	G : Moderate AM : White~pale yellowish brown SM : White~pale yellowish brown SP : Very pale yellowish brown	G : Moderate AM : Poor, white~light brownish gray SM : Colorless~pale yellowish brown SP : Almost colorless	G : Moderate AM : Poor, white SM : Colorless~white SP : None	G : Creamy~cream yellow AM : White~brownish white SM : Pale yellowish brown~Olive bistre SP : Faint, pale ochre
Glucose Czapek agar	G : Good AM : Pale brown SM : Brown~reddish brown SP : Brown~reddish brown	G : Good AM : Moderate. white~pale yellowish gray SM : Pale yellowish brown~dull yellow SP : Dull yellow	G : Good AM : Brownish white SM : Brown SP : Brown	G : Cream~orange buff AM : White~brownish white SM : Buff~grain SP : Orange buff
Sucrose Czapek agar	G : Good, wrinkled AM : Poor, white SM : Pale yellowish brown~yellowish brown SP : Yellowish brown	G : Moderate AM : Poor~scant SM : Pale yellowish brown SP : Pale yellowish brown	G : Good AM : Poor, white SM : White~brownish white SP : Pale reddish brown	G : Beige~cinnamon AM : White~brownish white SM : Rose beige~buff SP : Rose beige~buff
Glucose asparagin agar	G : Moderate AM : Moderate, white~light brownish gray SM : White~ivory SP : None	G : Moderate AM : Scant SM : Colorless~very pale yellowish brown SP : None	G : Poor AM : Scant~none SM : Colorless~white SP : None	G : Cream~orange buff AM : White~brownish white SM : Buff~grain SP : Orange buff
Morphology	Mainly mono-verticillus spira	Mainly spira	Mainly spira	Biverticillus spira

G : Growth, AM : Aerial mycelium, SM : Substratal mycelium, SP : Soluble pigment

streptomycetes isolated from various soil samples, and *Streptomyces* sp. No. 2-89 was newly isolated from a soil sample collected at Takatsuki City, Osaka, Japan. The strain No. 2-89 was very similar to *S. phaeoverticillatus* taxonomically, but did not form any secondary whirls as same as strain No. 1-125 or No. 613-15. Accordingly, these three strains were considered to belong to the same species of *Streptomyces*, and were designated as *Streptomyces phaeoverticillatus* var. *takatsukiensis*.

The strains belonging to *S. phaeoverticillatus* var. *takatsukiensis* were able to produce antimicrobial and antitumor antibiotics in the medium containing a sort of anthraquinone sulfonate compound. A new antibiotic was isolated from the culture filtrate of the strains of *S. phaeoverticillatus* var. *takatsukiensis* and was named kidamycin.

This paper deals with the new antimicrobial and antitumor antibiotic, kidamycin.

Table 2. Utilization of carbon sources of *Streptomyces phaeoverticillatus* and *S. phaeoverticillatus* var. *takatsukiensis* on PRIDHAM-GOTTLIEB agar medium

Carbon sources	*S. phaeoverticillatus* var. *takatsukiensis*			*S. phaeoverticillatus*[2)]
	No. 613-15	No. 1-125	No. 2-89	
D-Xylose	+	(−)	(−)	−
L-Arabinose	+	(−) (trace)	(−)	+
D-Glucose	+	−	(−) (none)	+
Sucrose	(−)	(−) (none)	(−) (none)	(−)
D-Galactose	+	(−) (trace)	+	+
L-Rhamnose	+	− (trace)	+	+
Raffinose	+	− (poor)	+	+
Lactose	+	(−) (poor)	+	+
D-Fructose	+	+	+	+
Maltose	+	(−)	+	(+)
Mannitol	(−)	+ (none)	+	+
D-Sorbitol	+	+ (poor)	(−)	+
L-Sorbose	−	−	−	
Dulcitol	−	−	−	(−)
Inositol	−	(−)	(−)	+
Inuline	−	(−) (none)	(−)	+
Salicin	±	−	−	±
Mannose	(−)	(−) (none)	+	+
Starch	+	+	+	+
Na-Acetate	±	−	−	(+)
Na-Citrate	±	−	−	(+)
Na-Succinate	±	−	−	±
none	−	−	−	±

Strains were incubated for 20 days at 27~28℃.
+=good growth; (−)=faint growth; ±=variable growth; −=no growth.
( ): formation of aerial mycelium, no ( ): good or moderate formation of aerial mycelium.

Table 3. The antimicrobial spectrum of kidamycin

Test organisms	Minimum inhibitory concentration (mcg/ml)
1 *Staphylococcus aureus* FDA 209 P	1.56
2 *Staphylococcus albus*	1.56
3 *Micrococcus flavus*	0.19
4 *Sarcina lutea* ATCC 9341	0.39
5 *Bacillus subtilis* PCI 219	1.56
6 *Bacillus cereus* ATCC 9634	0.78
7 *Corynebacterium sepedonicum*	0.78
8 *Escherichia coli* B	>100
9 *Shigella flexneri* KOMAGOME III	>100
10 *Pseudomonas aeruginosa*	>100
11 *Proteus vulgaris*	>100
12 *Brucella melitensis*	1.56
13 *Salmonella enteritidis* No. 11	100
14 *Klebsiella pneumoniae*	100
15 *Serratia marcescens*	100
16 *Streptococcus pyogenes* T-1	1.56
17 *Diplococcus pneumoniae* DP-1	0.39
18 *Mycobacterium tuberculosis* 607	1.56
19 *Lactobacillus fermenti* 36	0.19
20 *Candida albicans* YU-1200	>100
21 *Cryptococcus neoformans*	>100
22 *Aspergillus niger*	>100
23 *Trichophyton interdigitale*	>100

Taxonomy

As described above, all strains belonged to *S. phaeoverticillatus* var. *takatsukiensis* do not form any secondary whirls differing from *S. phaeoverticillatus*. Some cultural properties of strains No. 1-125, No. 613-15 and No. 2-89* differing from *S. phaeoverticillatus* are shown in Tables 1 and 2, but other properties are not distinguishable.

Fermentation

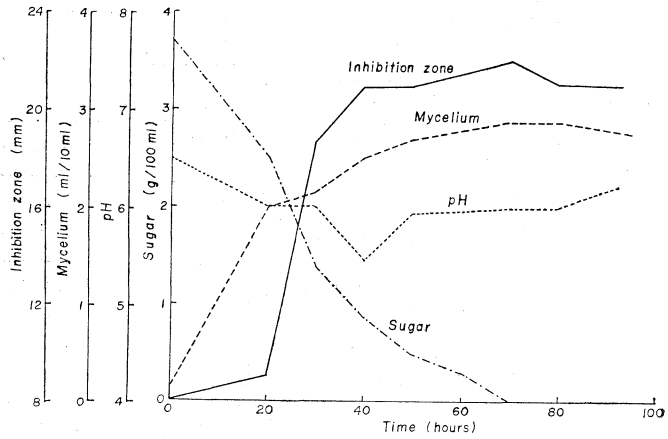
Large scale-fermentation of kidamycin was carried out according to the following procedure.

One thousand liter of fermentation medium consisting of 4% (w/v) glucose, 0.5% peptone, 0.3% dry yeast, 0.5% meat extract, 0.5% NaCl, 0.6% $CaCO_3$ and 0.5% Na-anthraquinone-2,7-disulfonate (pH 7.0) was placed in a 1,500-liter fermentor, and

* No. 613-15 : ATCC 21395, No. 1-125 : ATCC 21396, No. 2-89 : ATCC 21397

sterilized at 120°C for 30 minutes. After sterilization, the medium was inoculated with 100-liter preculture of *S. phaeoverticillatus* var. *takatsukiensis* No. 613-15, and cultivated at 28~30°C under aeration and agitation. A typical course of the fermentation was summarized in Fig. 1.

Fig. 1. Fermentation of kidamycin.

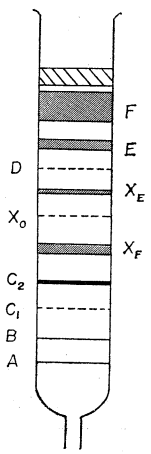


Details of additional substances such as Na-anthraquinone-2, 7-disulfonate and their roles in the fermentation will be reported elsewhere.

Isolation and Purification

After 92 hours of cultivation, the broth was filtered with filter aid, and the filtrate was extracted with chloroform at pH 8.6. The chloroform layer was washed with water, dried with Na_2SO_4, and the solvent was removed under vacuum. The residue obtained was washed with petroleum ether and dried to yield about 300 g of crude powder from 1,000 liters of the filtrate. The crude powder was further purified by column chromatography. As an example, silica gel column chromatography was carried out according to the following procedure: Silica gel (Mallinckrodt silisic acid, 100 mesh) was slurried with a mixture of benzene and methanol (4 : 1). The crude powder dissolved in the same solvent mixture was applied to the column, and the same solvent mixture was used as an eluant. A typical column chromatogram is shown in Fig. 2. Each fraction was eluted off in the order of A to F. The eluate of fraction F collected by an automatic fraction collector was checked by development of a bluish-purple color with nickel acetate (in methanol), pooled and then evaporated under vaccum to dryness. The residue obtained was dissolved in a small volume of ethyl acetate, and petroleum ether was then added for precipitation. The resultant precipitate which separated was washed with petroleum ether and dried. The crude kidamycin was obtained as an amorphous powder. Crude kidamycin was dissolved in chloroform and re-chromatographed on an activated alumina column using chloroform as an eluant. Orange-colored eluates were collected, and these were evaporated under vacuum to dryness. Crystallizing the residue from

Fig. 2. Column chromatogram of crude powder.
Adsorbent: Silica gel
Solvent: Benzene-methanol (4 : 1)



methanol, pure kidamycin was obtained as orange red needle crystals. According to this process, 35 g of pure kidamycin was obtained from 300 g crude powder.

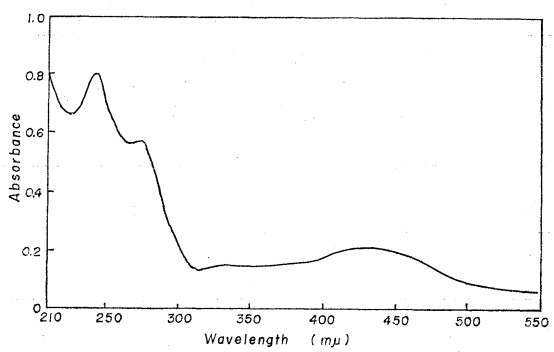
Physical and Chemical Properties

Kidamycin forms orange red needle crystals, melting at 214~217°C with decomposition (crystallized from methanol). Elemental analysis gave C 67.04 %, H 7.12 %, N 4.15 %, with no halogen and sulfur. The specific rotation was $[\alpha]_D^{20}$ +456.7° (*c* 1.5, chloroform). The ultraviolet absorption spectrum in methanol is characterized by maxima at 244 mμ ($E_{1cm}^{1\%}$ 807), 434 mμ ($E_{1cm}^{1\%}$ 210) and a shoulder at 270 mμ as shown in Fig. 3. The infrared absorption spectrum in KBr shows the characteristics bands at 1620, 1610, and 1585 cm^{-1} as illustrated in Fig. 4. The nuclear magnetic resonance spectrum of kidamycin in $CDCl_3$ indicates the presence of N-methyl groups, aromatic and aliphatic hydrogenes as shown in Fig. 5.

Kidamycin is soluble in chloroform, ethylene dichloride, ethyl acetate, *n*-butyl acetate, benzene, acetone, pyridine, dioxane, methanol, ethanol and aqueous acid, but is insoluble in petroleum ether, ligroin, *n*-hexane and water.

Positive color reactions are as follows: purple with NaOH in methanol, orange red with H_2SO_4, bluish purple with magnesium acetate in methanol, bluish purple with nickel acetate in methanol, and yellowish brown with ferric chloride in methanol. FEHLING, MOLISCH, and ninhydrin reactions are negative.

Fig. 3. Ultraviolet absorption spectrum of kidamycin (10 μg/ml methanol).



Rf values by ascending paper chromatography on Toyo No. 50 paper are as follows: 0.14 with acetonitrile, 0.71 with *n*-butyl acetate - dibutyl ether (3 : 1), and 0.68 with *n*-butanol - methanol - water (4 : 1 : 2). On thin-layer chromatography the Rf values are as follows: 0.81 with ethanol - 14 % ammonia water (4 : 1), 0.06 with ethanol - pyridine (4 : 1), using silica gel (Merck

Fig. 4. Infrared absorption spectrum of kidamycin (KBr).

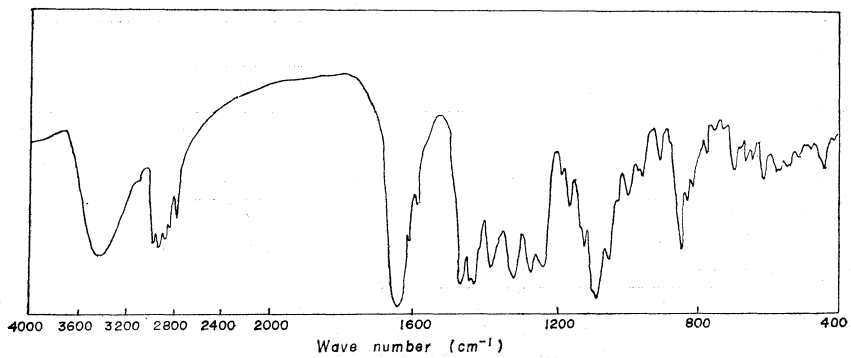


Fig. 5. NMR spectrum of kidamycin (crystallized from MeOH) in $CDCl_3$ (100 MHz).

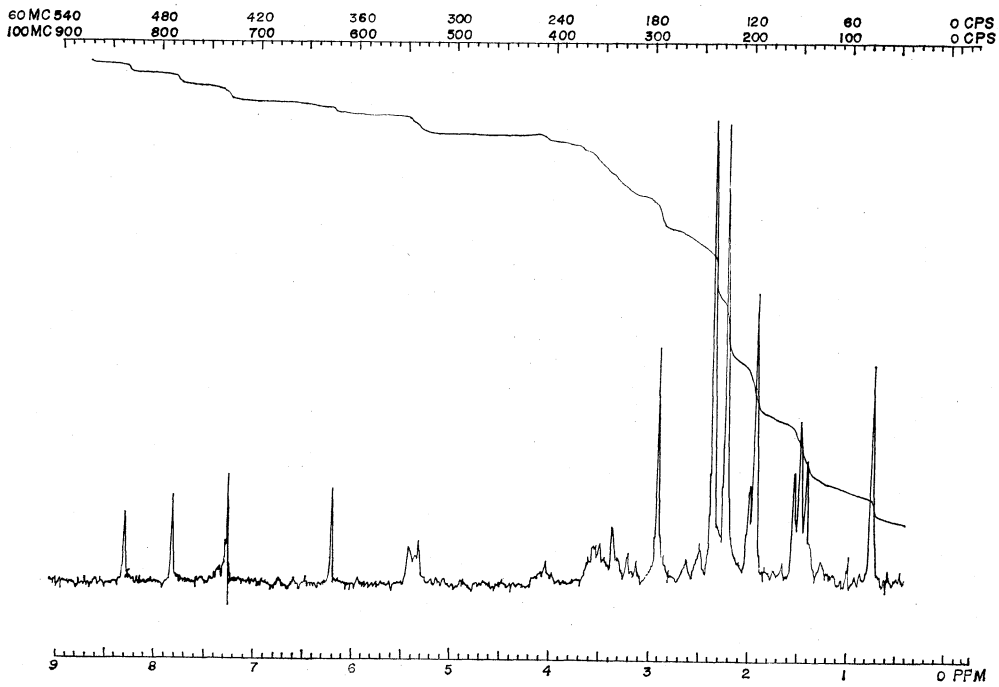


Table 4. Comparison of kidamycin and its related antibiotics

Antibiotic	Melting point (°C)	Ultraviolet absorption λ_{max} ($E^{1\%}_{1cm}$)	Elementary analysis (N %)	Molecular weight	Infrared absorption
Kidamycin	211.5~213 (decomp.) (from CH_3CN) 214~217 (from MeOH)	244 mμ (807), 270 mμ (s), 434 mμ (210)	N, 4.15	670~680 (from NMR & anal. data)	1645, 1610, 1590 cm^{-1}
Iyomycin B_1	>270	243~244 mμ (500), 270 mμ (330), 430 mμ (96)	N, 3.89	1100	
Iyomycin B_4	>270	243~244 mμ (360), 265 mμ (250), 430 mμ (59)	N, 8.94		
Hedamycin	243~245 (decomp.)	245 mμ (800), 260~265 mμ (s), 430 mμ	N, 3.76	748	1660, 1630 cm^{-1}
Rubiflavin		244 mμ, 265 mμ (s), 395 mμ (s), 428 mμ, 446 mμ (s)	N, 3.45	412	
Anthracidin A	114~116	233 mμ (784), 358 mμ (465)	N, 3.91	452	
Anthracidin B	111~112.5	233 mμ (747), 350 mμ (390)	N, 3.69	397	
Pluramycin A	200~215 (darkening) (from EtOAC)	245 mμ (672), 265~270 mμ (s)	N, 3.66		1744, 1658, 1625, 1581, 1233 cm^{-1}
	177 (darkening) (from EtOH)		N, 3.80		
Neopluramycin	180~184 (decomp.)	216 mμ (640), 243 mμ (790), 270 mμ (564), 430 mμ (164)	N, 3.93	750±35	1745, 1645, 1605 cm^{-1}

(s): Shoulder

Kieselgel G), and about 0.1 with water-saturated ethyl acetate using aluminium oxide paper (Schleicher and Schüll No. 288).

Biological Properties

Kidamycin is an antitumor antibiotic which has inhibitory activities against a variety of microorganisms, particularly Gram-positive bacteria. The antimicrobial spectrum of kidamycin was observed by agar two-fold serial dilution method. Table 3 shows the minimum inhibitory concentrations of kidamycin against various microorganisms. The acute LD_{50} of kidamycin in mice is 12.5~20.0 mg/kg when given either intravenously or intraperitoneally. Kidamycin exhibited significant inhibitory activity on experimental tumors. Details of the antitumor experiments will be reported elsewhere.

Discussion

The differential points of kidamycin from other antitumor antibiotics are summarized in Table 3. Pluramycin A[5], iyomycin B_1[3], and iyomycin B_4[3] have no clear melting or decomposition points. Hedamycin[6] melts at 243~245°C with decomposition. The melting points of anthracidin A[7] and anthracidin B[7] are 114~116°C and 111~112.5°C respectively; that of rubiflavin is uncertain, while kidamycin melts at 214~217°C with decomposition. The infrared absorption spectrum of hedamycin shows bands at 1630 and 1660 cm^{-1}, characteristic for carbonyl groups, pluramycin shows bands at 1630, 1660 and 1745 cm^{-1}, and kidamycin shows bands at 1610 and 1645 cm^{-1}. The ultraviolet absorption spectrum of rubiflavin[8] shows maxima at 244 and 428 mμ, and shoulders at 165, 395 and 446 mμ, while kidamycin shows maxima at 244 and 434 mμ, and a shoulder at 270 mμ. Neopluramycin[9] is distinguished from kidamycin by its melting point and by their infrared absorption spectra.

As the results of the differentiation mentioned above, kidamycin was confirmed to be a new antibiotic having antitumor and antimicrobial activities.

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